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PATENTS

## IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of	BOX AF
Catherine HANNI et al.	Confirmation No. 6537
Serial No. 09/423,259	GROUP 1655
Filed March 2, 2000	Examiner J. Einsmann

METHOD FOR DETECTING THE PRESENCE  
OF BIOLOGICAL MATTERS OF BOVINE  
ORIGIN, AND OLIGONUCLEOTIDES FOR  
ITS IMPLEMENTATION

DECLARATION UNDER RULE 132

Commissioner for Patents  
Washington, D.C. 20231

Sir:

I, Vincent LAUDET, hereby declare as follows:

My relevant background and experience are set forth on the attached C.V. I make this declaration in support of the present application, and to provide evidence in rebuttal of several contentions set forth in the Official Action of December 21, 2001. I further declare that one of ordinary skill in the art would not find the present invention obvious in view of LOFTUS et al. in view of FEI et al.

In the outstanding Official Action, it was alleged that the present application fails to provide evidence of unexpected results showing that the claimed probes and primers have the properties asserted by the applicants. However, I further declare that the present application and results set forth in the

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application clearly support the properties described in the present invention.

Indeed, the present application contains figures showing that SEQ ID 3 and SEQ ID 6 are able to specifically amplify beef DNA and not DNA from other species such as sheep, pig, horse, chicken, duck and turkey.

We also present further evidence of the unexpected properties exhibited by the present invention, evidence showing that in addition to SEQ ID 3 with SEQ ID 6, other primer pairs also exhibit these unexpected properties. All of these primer pairs allow the detection of beef DNA but not DNA from other species such as sheep, pork, horse, chicken, human.

Figures 1-6 as shown below illustrate the agarose gel results of the primer pairs of SEQ ID NO. 3 with SEQ ID NO. 6; SEQ ID NO. 11 with SEQ ID NO. 6; SEQ ID NO. 3 with SEQ ID NO. 12; SEQ ID NO. 12 with SEQ ID 14; SEQ ID NO. 13 with SEQ ID NO. 14; and SEQ ID NO. 9 with SEQ ID NO. 10. The data clearly show that the primers of the present invention are unique tools that specifically test the presence of biological matter of bovine origin. In all cases, a clear band at the expected size (size range 163 to 498 bp) is obtained. In a unique case (SEQ ID 12-SEQ ID 14; see Figure 4), a shorter band found only in beef is also detected with the expected band.

In addition to using the hybridization procedure described in the present application, we clearly show (see

Figures 9-11) that the PCR fragments (SEQ ID 8 and SEQ ID 15 to 17) can specifically hybridize with various set of probes derived from SEQ ID 7, SEQ ID 15, SEQ ID 17, and SEQ ID 19 (Figures 9-11). This clearly demonstrates the ability of our process to detect the presence of biological matters of bovine origin.

We would like to point out several factors that strongly suggest that the primer pairs of the present invention are unique and non-obvious.

First, we do not propose only one specific pair of primers. We provide a relatively large series of primers that can be used in a variety of combinations (for example SEQ ID 3 can be used either with SEQ ID 6 or SEQ ID 12 or SEQ ID 6 can be used with either SEQ ID 3 or SEQ ID 11). This is advantageous because this allows detecting the presence of beef DNA from a number of sources (i.e. fresh meat or extremely degraded DNA). The settling of PCR primers that allow detection of degraded DNA from processed food is certainly not a routine optimization.

Second, it is clear from the literature (see the primers cited in the LOFTUS et al. paper cited by the Official Action) that numerous pairs of primers do not allow the specific detection of beef and, in contrast it would be expected that they cross-hybridize with DNA from other species. Once again, we show that the primer design and primer test is fully inventive.

Third, the primers, PCR fragments, and probes described in the present application have indeed unexpected properties.

They all are strictly specific for beef. The primers (for example SEQ 1 to 6 and SEQ ID 9 to 14) only allow the amplification of beef DNA, not DNA from other species. The generated PCR fragments (for example SEQ ID 8 and SEQ ID 15 to 17) are indeed beef specific and can be easily recognized from other species by DNA sequencing or by hybridization with specific probes of known beef origin. The Examiner's attention is also respectfully directed to Figures 7-9, shown below. The probes (either SEQ ID 7 and SEQ ID 19 or probes derived from the PCR fragments SEQ ID 15 to 17) are beef specific and do not recognize PCR fragments which amplify DNA from other species. Taken together, our results clearly show that the present invention is not the result of routine optimization but was created during an extensive inventive process.

Finally, the Official Action stated that the paper from FEI et al. represents a previous disclosure of our method. We respectfully disagree with this statement. As shown below in Figs. 7 and 8, the primers from FEI et al. contrast with our own primers and are not specific to beef.

When tested in the same conditions as our primers (hybridization temperature at 62°C), the FEI et al. primers generate several non-specific bands or cross-hybridization results for DNA from beef, sheep, horse or pork. On the same conditions, our primers are specific for beef and they do not amplify DNA not of bovine origin.

When tested at 66°C, which represents a very high, and unusual hybridization temperature (the one used in FEI et al., 1996), the primers also generate unspecific PCR fragments (see stars in Fig. 7). The use of such a high hybridization temperature gives rise to an inefficient process for the detection of beef DNA. In fact, it is well known that a prolonged action of temperature has a negative impact on PCR yield. Indeed, FEI et al. claimed that only 0.5 ng of beef DNA can be detected in an excess of 500 ng of pork DNA. In contrast, with our primers, we have found that our primers are more efficient. Our primers can detect much lower amounts of beef DNA even in a sample containing an excess DNA from other species.

Amount of beef DNA	Amount of pork DNA	Primer pair	Detection of a beef specific PCR fragment
25 ng	60 ng	SEQ ID 3-6	+
5 ng	60 ng	SEQ ID 3-6	+
0.5 ng	60 ng	SEQ ID 3-6	+
0.05 ng	60 ng	SEQ ID 3-6	+
0.005 ng	60 ng	SEQ ID 3-6	+
0.0005 ng	60 ng	SEQ ID 3-6	-
0 ng	60 ng	SEQ ID 3-6	-
25 ng	60 ng	SEQ ID 13-14	+
5 ng	60 ng	SEQ ID 13-14	+
0.5 ng	60 ng	SEQ ID 13-14	+
0.05 ng	60 ng	SEQ ID 13-14	+
0.005 ng	60 ng	SEQ ID 13-14	-
0.0005 ng	60 ng	SEQ ID 13-14	-
0 ng	60 ng	SEQ ID 13-14	-
25 ng	60 ng	FEI BR-BF	+
5 ng	60 ng	FEI BR-BF	+
0.5 ng	60 ng	FEI BR-BF	+
0.05 ng	60 ng	FEI BR-BF	-*
0.005 ng	60 ng	FEI BR-BF	-*
0.0005 ng	60 ng	FEI BR-BF	-*
0 ng	60 ng	FEI BR-BF	-*

**Table 1 :** Comparison of the efficiency of beef detection with various primer pairs  
 + : PCR fragment at the expected size ; - : no PCR fragment detected at the expected size.  
 \* : In the case of the FEI BF and FEI BR primers it is impossible to specifically detect beef versus pork (see Figure 7 and 8, lane 4) and thus the absence of detection is inferred by comparison with the signal observed using pork DNA alone.

It is noted that FEI et al. tested their primers in a relatively narrow range of species (beef, sheep, pork and chicken). This may explain why they did not report cross-hybridization bands. In our case, we consistently tested for a variety of species. For example, we systematically used human DNA as a control, since humans are a classical source of contamination in PCR.

In the analysis of FEI et al., the ability of the primers to discriminate for beef DNA mixed with DNA from other species was tested. These authors claimed to be able to detect 0.5 ng of beef DNA mixed in 500 ng of pork DNA. In our conditions, it is difficult to perform PCR experiments with such a high amount of DNA. Thus, we have compared the ability of two of our PCR pairs (SEQ ID 3-SEQ ID 6 and SEQ ID 13-SEQ ID 14) to detect a low amount of beef DNA in a large excess of pork DNA with the FEI et al. primers. We observed (see Fig. 12 and Table 1) that our primers are able to detect up to 0.005 ng of beef DNA mixed with 60 ng of pork DNA, whereas the FEI et al. primers are limited by a much less sensitive detection. In fact, as the FEI et al. primers are not specific, it is difficult to precisely assess their reliability.

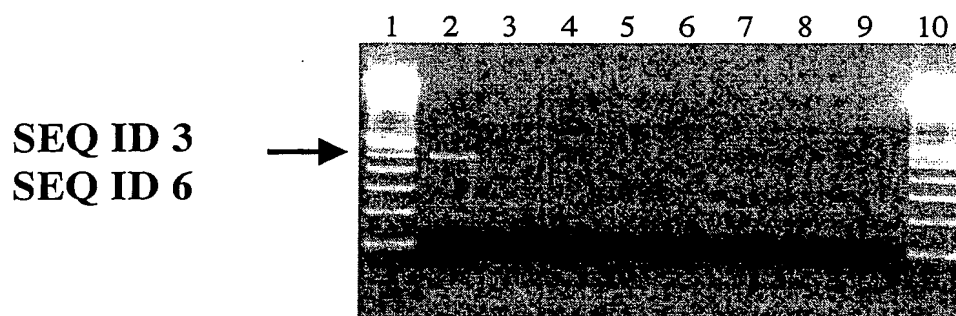
Nevertheless, with an estimate of the intensity of the PCR bands, we can deduce that the FEI et al. primers cannot detect much more than 0.5 ng of beef DNA in 60 ng of pork DNA. This value is in accordance with the data of FEI et al. Thus,

our PCR primers allow for a much more sensitive detection of beef DNA than the FEI et al. primers. This observation is consistent with the unusual base composition of the sequence targeted by FEI et al. primers.

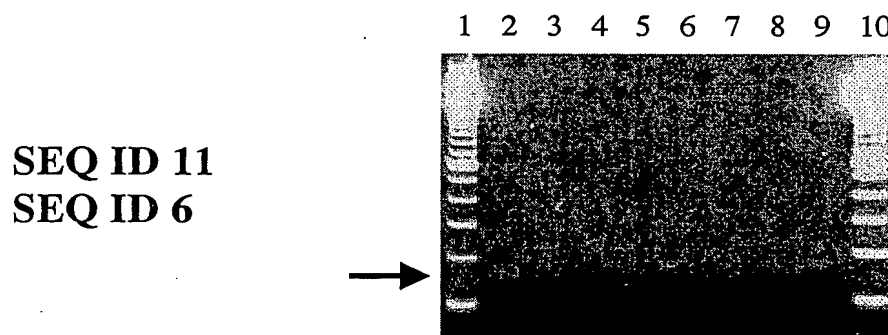
Thus, the FEI et al. primers are not specific and not efficient. Thus, the primers of FEI et al. cannot be considered in any way as a previous disclosure of our method.

Figures 1-12 are shown as follows.



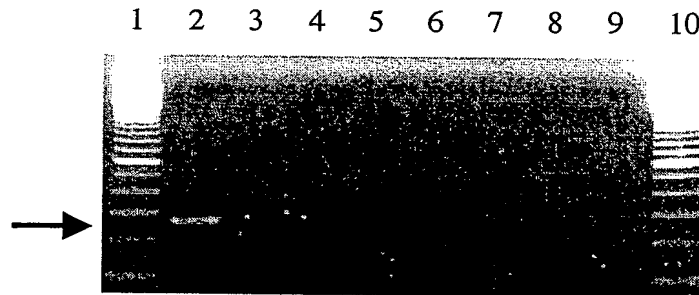


**Figure 1** : The pair of primer SEQID3 and SEQID6 allows specific detection of beef sequences (lane 2) in contrast to sheep (lane 3), pork (lane 4), horse (lane 5), chicken (lane 6), and human (lane 7). The lanes 1 and 10 are molecular size markers and the lanes 8 and 9 are empty controls testing possible contamination during PCR amplification (lane 8) or handling of the reagents in the PCR room (lane 9). The size of the expected band is 498 bp.  
PCR conditions : 1mM MgCl<sub>2</sub>, hybridization temperature at 60°C, 30 cycles.



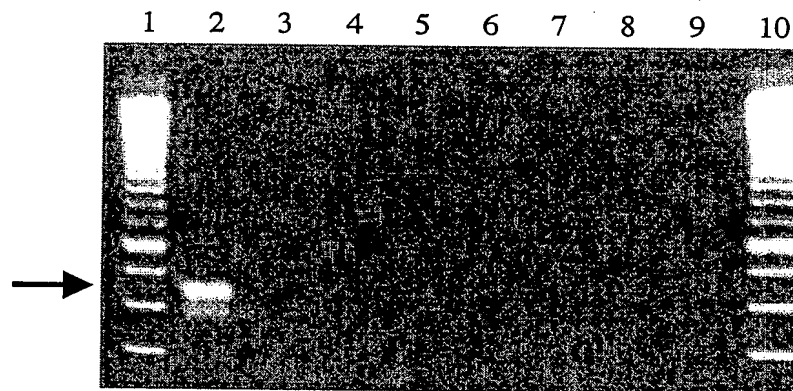
**Figure 2** : The pair of primer SEQID11 and SEQID6 allows specific detection of beef sequences (lane 2) in contrast to sheep (lane 3), pork (lane 4), horse (lane 5), chicken (lane 6), and human (lane 7). The lanes 1 and 10 are molecular size markers and the lanes 8 and 9 are empty controls testing possible contamination during PCR amplification (lane 8) or handling of the reagents in the PCR room (lane 9). The size of the expected band is 163 bp.  
PCR conditions : 1mM MgCl<sub>2</sub>, hybridization temperature at 60°C, 30 cycles.

SEQ ID 3  
SEQ ID 12



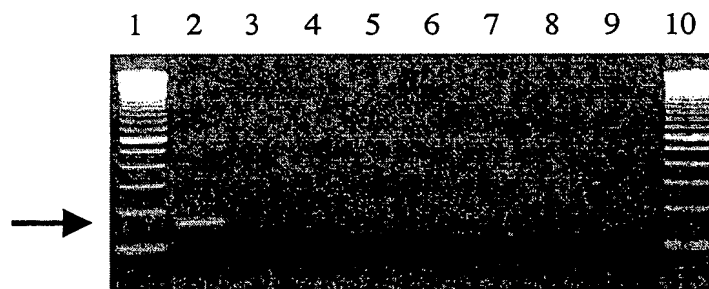
**Figure 3 :** The pair of primer SEQID3 and SEQID12 allows specific detection of beef sequences (lane 2) in contrast to sheep (lane 3), pork (lane 4), horse (lane 5), chicken (lane 6), and human (lane 7). The lanes 1 and 10 are molecular size markers and the lanes 8 and 9 are empty controls testing possible contamination during PCR amplification (lane 8) or handling of the reagents in the PCR room (lane 9). The size of the expected band is 258 bp.  
**PCR conditions :** 1mM MgCl<sub>2</sub>, hybridization temperature at 60°C, 30 cycles.

SEQ ID 12  
SEQ ID 14



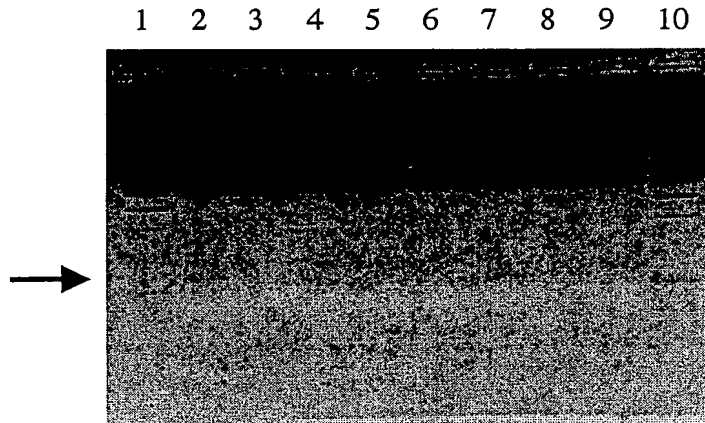
**Figure 4 :** The pair of primer SEQID12 and SEQID14 allows specific detection of beef sequences (lane 2, a non specific band is also observed ca. 50 bp below ; star) in contrast to sheep (lane 3), pork (lane 4), horse (lane 5), chicken (lane 6), and human (lane 7). The lanes 1 and 10 are molecular size markers and the lanes 8 and 9 are empty controls testing possible contamination during PCR amplification (lane 8) or handling of the reagents in the PCR room (lane 9). The size of the expected band is 443 bp.  
**PCR conditions :** 1mM MgCl<sub>2</sub>, hybridization temperature at 60°C, 30 cycles.

SEQ ID 13  
SEQ ID 14

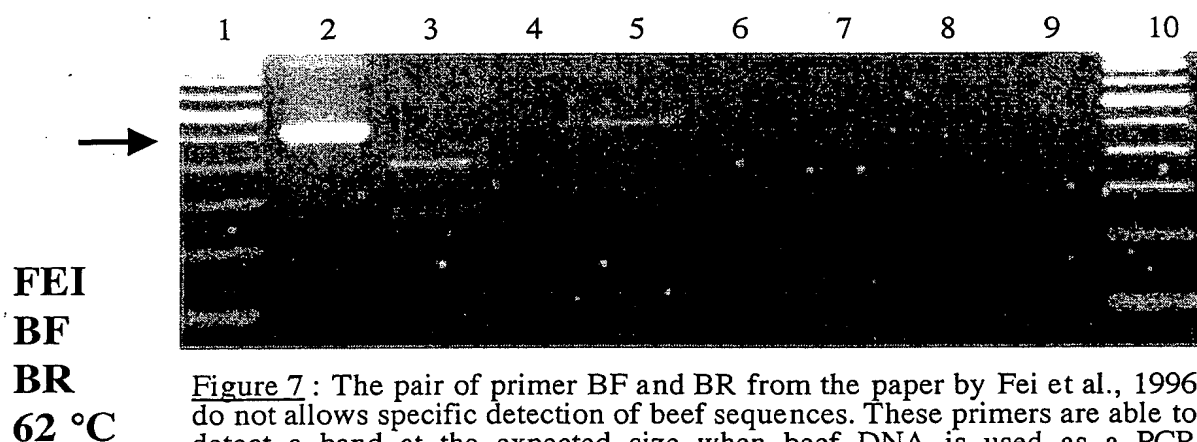


**Figure 5** : The pair of primer SEQID13 and SEQID14 allows specific detection of beef sequences (lane 2) in contrast to sheep (lane 3), pork (lane 4), horse (lane 5), chicken (lane 6), and human (lane 7). The lanes 1 and 10 are molecular size markers and the lanes 8 and 9 are empty controls testing possible contamination during PCR amplification (lane 8) or handling of the reagents in the PCR room (lane 9). The size of the expected band is 172 bp.  
PCR conditions : 1mM MgCl<sub>2</sub>, hybridization temperature at 65°C, 30 cycles.

SEQ ID 9  
SEQ ID 10

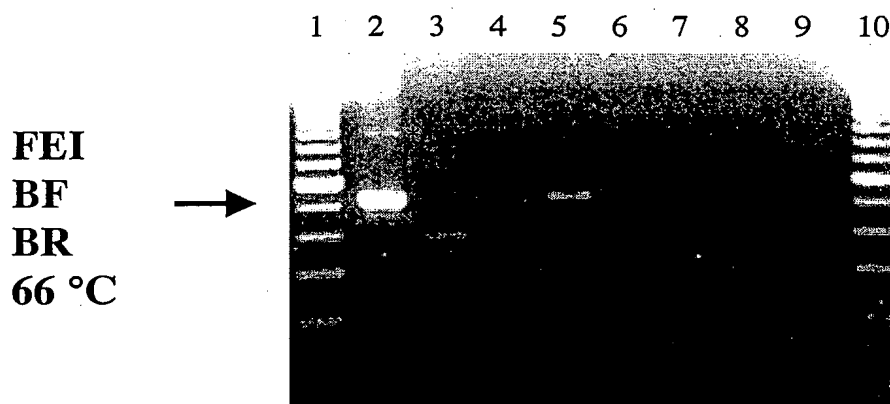


**Figure 6** : The pair of primer SEQID9 and SEQID10 allows specific detection of beef sequences (lane 2) in contrast to sheep (lane 3), pork (lane 4), horse (lane 5), chicken (lane 6), and human (lane 7). The lanes 1 and 10 are molecular size markers and the lanes 8 and 9 are empty controls testing possible contamination during PCR amplification (lane 8) or handling of the reagents in the PCR room (lane 9). The size of the expected band is 174 bp.  
PCR conditions : 2mM MgCl<sub>2</sub>, hybridization temperature at 58°C, 30 cycles.



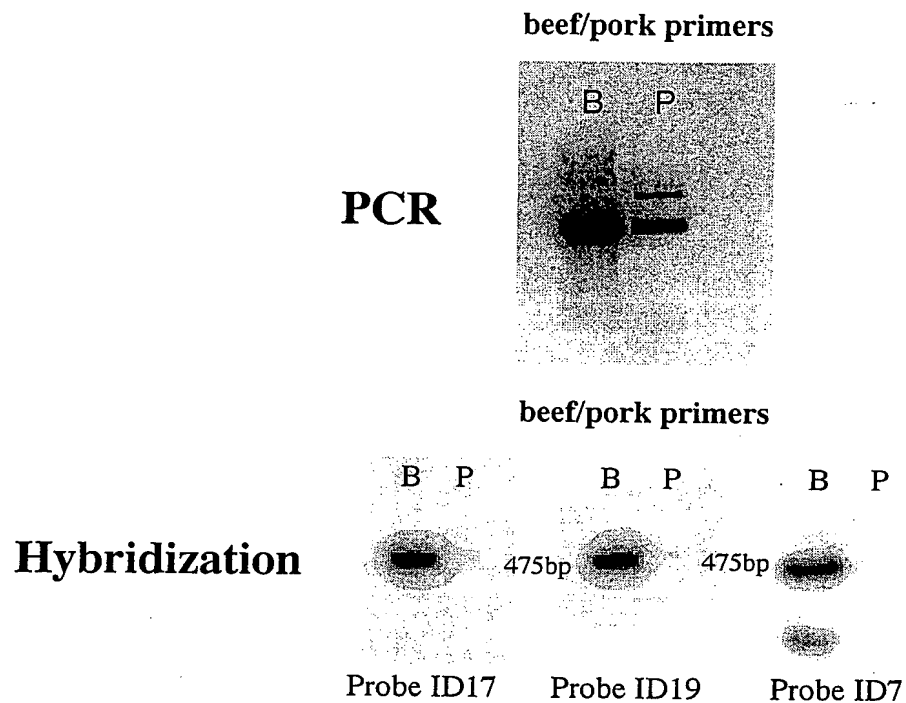
**Figure 7 :** The pair of primer BF and BR from the paper by Fei et al., 1996 do not allows specific detection of beef sequences. These primers are able to detect a band at the expected size when beef DNA is used as a PCR template (lane 2) but a non specific band is also observed at ca. 800 bp (star on lane 2). In contrast to the data reported in Fei et al., 1996 non specific bands are also observed when template DNA is the following species sheep (stars on lane 3), pork (star on lane 4) or horse (star on lane 5). As expected no bands are detected when DNA is from chicken (lane 6) or human (lane 7). The lanes 1 and 10 are molecular size markers and the lanes 8 and 9 are empty controls testing possible contamination during PCR amplification (lane 8) or handling of the reagents in the PCR room (lane 9). The size of the expected band is 547 bp.

PCR conditions : 2mM MgCl<sub>2</sub>, hybridization temperature at 62°C, 30 cycles.



**Figure 8 :** The pair of primer BF and BR from the paper by Fei et al., 1996 do not allows specific detection of beef sequences. These primers are able to detect a band at the expected size when beef DNA is used as a PCR template (lane 2) but a non specific band is also observed at ca. 800 bp (star on lane 2). In contrast to the data reported in Fei et al., 1996 non specific bands are also observed when template DNA is the following species sheep (stars on lane 3), pork (star on lane 4) or horse (star on lane 5). As expected no bands are detected when DNA is from chicken (lane 6) or human (lane 7). The lanes 1 and 10 are molecular size markers and the lanes 8 and 9 are empty controls testing possible contamination during PCR amplification (lane 8) or handling of the reagents in the PCR room (lane 9). The size of the expected band is 547 bp.

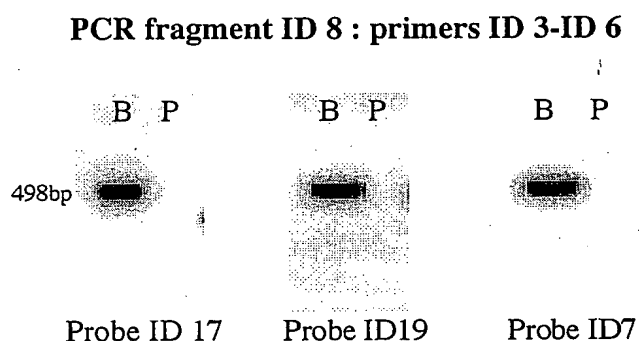
PCR conditions : 2mM MgCl<sub>2</sub>, hybridization temperature at 66°C, 30 cycles.



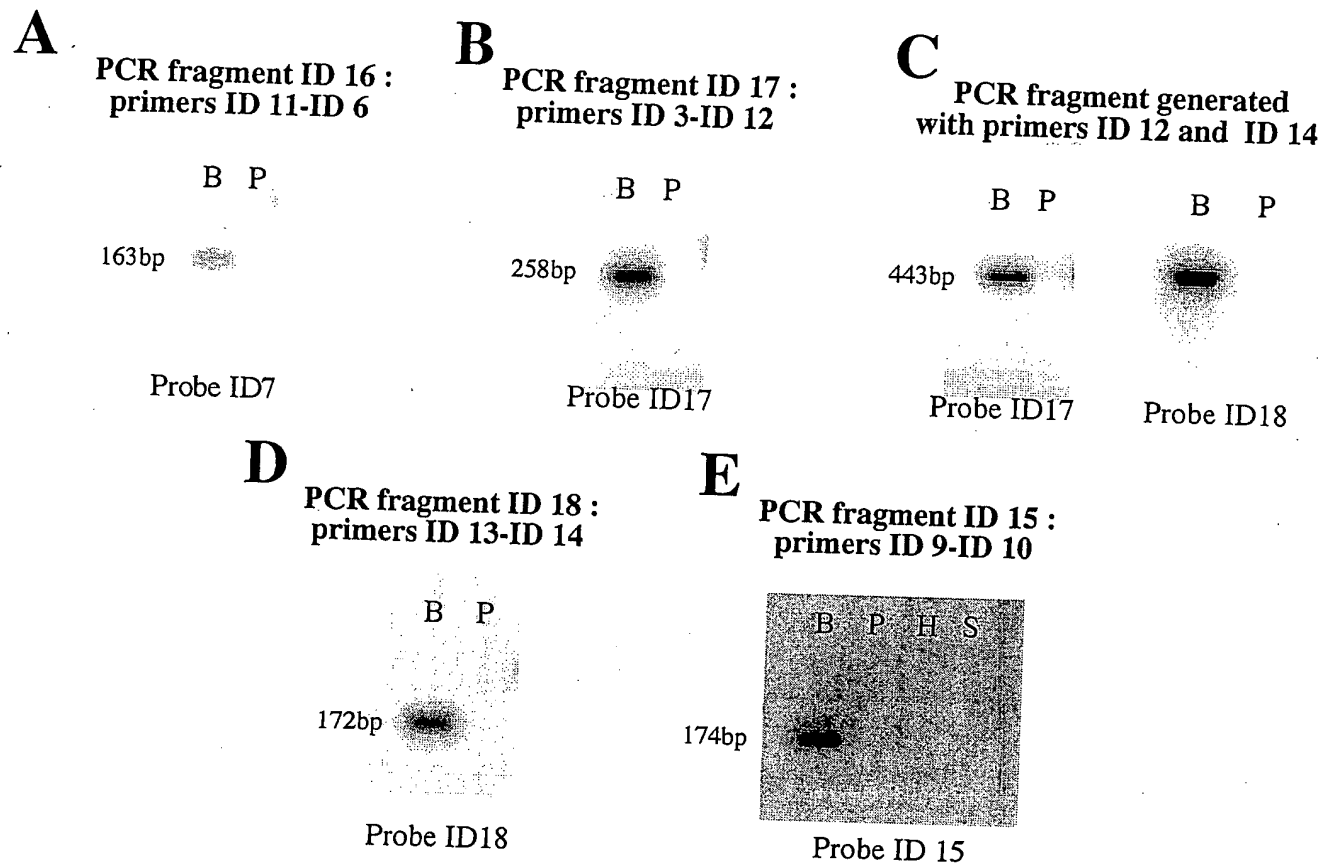
**Figure 9** : The probes derived from SEQ ID 17, SEQ ID 19 and SEQ ID 7 are beef-specific.

**Upper panel** : A PCR was performed with non specific primers able to amplify both beef (B) and pork (P) DNA. This PCR generates a 475 fragment for both species, as expected. 5' beef/pork primer : ATTGACTGTACATAGTACAT ; 3' beef/pork primer : ATGTCCTGTGACCATTGACTG. This fragment encompasses the regions recognized by probes derived from SEQ ID 17, SEQ ID 19, SEQ ID 7.

**Lower panel** : The PCR fragments from the upper panel were transferred on a nitrocellulose membrane and the membrane was hybridized with probes derived from either SEQ ID17 (left), SEQ ID 19 (middle) or SEQ ID7 (right). A clear beef-specific band is observed, clearly showing the specificity of the probes.

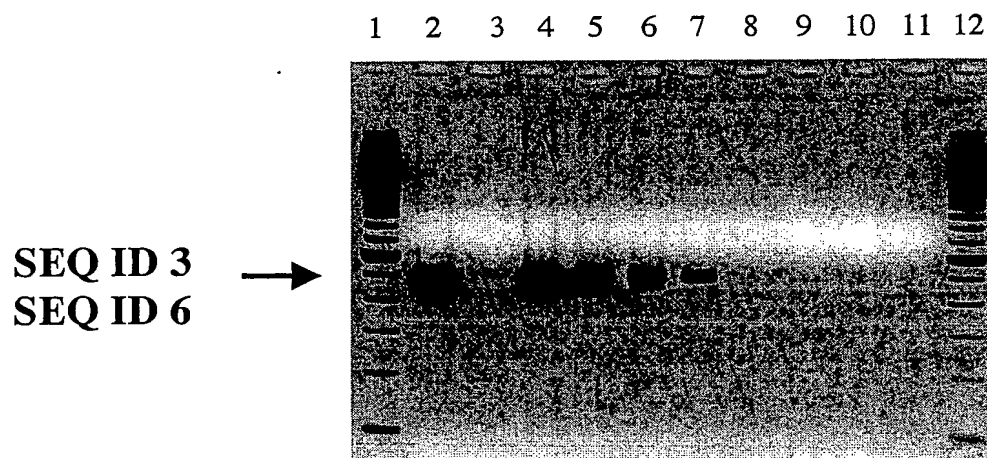


**Figure 10** : Specific hybridization of beef-specific PCR fragments with beef-specific probes. The pair of primers SEQ ID 3 and SEQ ID 6 generates the PCR fragment SEQ ID 8. This fragment is beef specific since it cannot be obtained using pork DNA as a template (see Fig. 1). Furthermore, this fragment is specifically recognized by the probes derived from SEQ ID 17, SEQ ID 19 and SEQ ID 7. B : Beef DNA, P : Pork DNA



**Figure 11** : Specific hybridization of beef-specific PCR fragments with beef-specific probes.

- (A) The pair of primers SEQ ID 11 and SEQ ID 6 generates the PCR fragment SEQ ID 16. This fragment is beef specific since it cannot be obtained using pork DNA as a template (see Fig. 2). Furthermore, this fragment is specifically recognized by the probe derived from SEQ ID 7.
- (B) The pair of primers SEQ ID 3 and SEQ ID 12 generates the PCR fragment SEQ ID 17. This fragment is beef specific since it cannot be obtained using pork DNA as a template (see Fig. 3). Furthermore, this fragment is specifically recognized by the probe derived from SEQ ID 17.
- (C) The pair of primers SEQ ID 12 and SEQ ID 14 generates a beef-specific PCR fragment (see Fig. 4). Furthermore, this fragment is specifically recognized by the probes derived from SEQ ID 1 (left) or SEQ ID 18 (right).
- (D) The pair of primers SEQ ID 13 and SEQ ID 14 generates the PCR fragment SEQ ID 18. This fragment is beef specific since it cannot be obtained using pork DNA as a template (see Fig. 5). Furthermore, this fragment is specifically recognized by the probe derived from SEQ ID 18.
- (E) The pair of primers SEQ ID 9 and SEQ ID 10 generates the PCR fragment SEQ ID 15. This fragment is beef specific since it cannot be obtained using pork, horse or sheep DNA as a template (see Fig. 5). Furthermore, this fragment is specifically recognized by the probe derived from SEQ ID 15. (B : Beef DNA, P : Pork DNA, H : Horse DNA ; S : Sheep DNA).



**Figure 12 :** Specific detection of beef DNA mixed with a large excess of pork DNA. The PCR was performed using primers SEQ ID3 and SEQ ID 6 using the conditions described Figure 1. The size of the PCR product is 498 bp. Lanes 1 and 12 are size markers.

Lane 2 : Only Beef DNA (25ng) was introduced in the PCR reaction : a specific band is observed at the expected size

Lane 3 : Only Pork DNA (25ng) was introduced in the PCR reaction : no positive amplification is observed.

Lane 4 to 9 : decreasing amount of beef DNA is introduced in a fix amount (60ng) of pork DNA. The amount of beef DNA used is as follow : Lane 4, 25 ng ; Lane 5, 5 ng ; Lane 6, 0.5 ng ; Lane 7, 0.05 ng ; Lane 8, 0.005 ng ; Lane 9, 0.0005 ng. The detection limit in this assay is 0.005 ng of beef DNA in 60 ng of pork, i.e. 1 molecule of beef DNA for 12000 thousands molecules of pork. The band at the expected size is barely visible on lane 8. No band was detected on lane 9.

Lanes 10 and 11 : Blank controls to ensure that no contamination occurred during the PCR.



And I further declare that all statements made herein of my knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both under §1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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date

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